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Safe and sustainable systems for food-grade fermentations by genetically modified lactic acid bacteria

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Abstract

The last decade has seen a great increase in innovative improvements of lactic acid bacteria used in industrial food fermentations. In order to allow the genetically modified lactic acid bacteria to reach the market place, their novel genetic combinations should be selected, stably maintained, and expressed using food-grade systems that are safe, stable, and sustainable. This paper aims to review the food-grade systems that have been constructed with specific attention for self-cloning approaches, and focuses on recent developments on food-grade selection markers, chromosome inactivation, stabilization, and amplification strategies, as well as approaches for controlled gene expression.

Keywords: Lactic acid bacteria; Food-grade selection and induction systems; Self-cloning; Lactose metabolism

1. Introduction

The last decade has seen an impressive increase in the interest of the genetics of lactic acid bacteria used in industrial food fermentations. As a consequence, a variety of genetic systems have been developed that have reached a high degree of sophistication, particulary in *Lactococcus lactis* that has become the paradigm for lactic acid bacteria (Gasson & de Vos, 1994). These genetic systems have been valuable in the analysis of industrially important traits and their improvement by genetic, metabolic and protein engineering. In addition, various novel properties have been introduced in lactic acid bacteria (Daly, Fitzgerald & Davis, 1996; de Vos, Kleerebezem & Kuipers, 1997; de Vos et al., 1998). The resulting genetically modified lactic acid bacteria are now being developed for a variety of traditional, novel and innovative applications that relate to their use as starters of dairy and other food fermentations, probiotic and other health-designed cultures, or industrial hosts for the production of peptides, enzymes, or metabolites. Most of these improvements have been realized by exploiting the abundance of cloning, integration, and expression vectors that are based on the panoply of promiscuous plasmids present in *L. lactis* (Leenhouts & Venema, 1993; de Vos & Simons, 1994). In addition, vectors have been developed for other lactic acid bacteria based on their endogenous plasmids (Pouwels & Leer, 1993; Mercenier, Pouwels & Chassy, 1994). In order to select the appropriate transformants and maintain selective pressure on the genetic modification, these vectors were equipped with one or more genes coding for resistance to antibiotics, such as chloramphenicol, erythromycin, tetracycline or spectinomycin. It is evident that such improved lactic acid bacteria are instrumental in showing proof of concept but are not suitable to reach the market place. Transferable antibiotic resistance markers should not be present in microorganisms that are used in food applications, because of legal, ethical, and in some cases, also scientific reasons. Similar considerations relate to some of the inducible gene expression systems that are being widely studied in lactic acid bacteria at present (Kok, 1996). These have been developed into controlled protein production systems that are of great significance as their application allows uncoupling of growth and expression of desired genes (Kuipers, de Ruyter, Kleerebezem &

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de Vos, 1997). However, some of the first systems were triggered by undesired toxic environmental stimuli, such as mitomycin C (Nauta, van Sinderen, Karsens, Smit, Venema & Kok, 1996). An effective way to eliminate the obstacles related to the use of antibiotic resistance markers or toxic compounds, is to develop industrial strains that build on, so-called, food-grade systems. Such food-grade approaches were already proposed more than a decade ago for selection markers based on natural properties of lactic acid bacteria (de Vos, 1987). This paper aims to review the food-grade systems that have since been constructed and focuses on recent developments on food-grade selection markers, chromosomal inactivation, stabilization, amplification strategies and approaches for controlled gene expression.

2. Food-grade and self-cloning systems

There are various specifications that should be met by food-grade systems, independent to the question of whether they relate to selection markers, exploitation of the chromosome, or controlled gene expression strategies (Table 1). First and foremost, they should be safe, well-characterized, and stable as well as versatile. The aspect of safety should be addressed in the same way as other issues that relate to novel foods (CEC-258, 1997). The food-grade systems should be as safe as the host in which they are introduced. Hence, it is obvious that they need to be based on DNA from lactic acid bacteria or other microbes with a long history of safe use in the food industry. In addition, the food-grade systems need to be characterized with state-of-the-art technologies. These may range from the use of modern taxonomic approaches for the identification of donor and host to the confirmation of the genetic constructs by appropriate molecular techniques, including sequence analysis, PCR amplification, and DNA–DNA hybridization. Moreover, the application of the food-grade systems should result in genetically modified lactic acid bacteria that are sufficiently stable under industrial conditions, in the foods themselves, or following passage in the gastro-intestinal tract. It is for this reason that the genetic systems should have the desired stability or that selective pressure should be exercised at the crucial stages in these processes. Finally, it is evident that food-grade systems should be versatile and, thus, based on DNA fragments that are small, equipped with appropriate features that facilitate genetic handling and devoid of sequences that affect the fitness of the host. A second important criterion for food-grade systems is that they should contain no antibiotic resistance markers and be compatible with the desired food application. This implies in some cases that no additives may be used, even salt, in order to stay within the limits dictated by the product laws. A third factor that relates to the sustainability of food-grade systems is preventing the use of harmful compounds to sustain safety and reduce environmental load. This applies for instance to avoiding the use of heavy metals, although promising selection systems may be based on resistances against these compounds (Liu, Leelawatcharamas, Harvey & Dunn, 1996). Finally, all systems need to be applied in industrial settings or in food products in an efficient, timely, and cost-effective manner. This strongly limits the range of possibilities since, for instance, it should be possible to apply and maintain the proper temperature upshift in a large-scale fermentation when a thermo-inducible gene expression system is used or, in the case of a sugar-based selection marker, to incorporate the specific sugar at the right concentration at the right time in a food fermentation.

Table 1
Criteria for sustainable food-grade systems

Safe as host and highly characterized, stable and versatile
Food-compatible and devoid of antibiotic resistance markers
Preventing use of harmful compounds
Applicable on industrial scale or in food products

A particular situation is encountered when self-cloning approaches are considered that are by definition food-grade when applied to lactic acid bacteria. Self-cloning systems are well-defined and are the simplest form of genetic modification, i.e. the re-introduction of DNA from a host that is modified or from a closely related strain of the same species. Provided the host is non-pathogenic, self-cloning is excluded from the EU Directive on the contained use of genetically modified micro-organisms (CEC-219, 1990). Consequently, lactic acid bacteria obtained by self-cloning can be used in industrial fermentations under the same conditions as for the non-modified parental strains avoiding the need for dedicated installations or procedures. Hence, products that are free from viable cells can be commercialized without biosafety considerations although they will be subject to the EU Directive on Novel foods and Novel additives (CEC-258, 1997). Paradoxically, self-cloning is not excluded from the EU Directive on the deliberate release of genetically modified microorganisms (CEC-220, 1990). However, it has been predicted that products obtained by self-cloning of lactic acid bacteria were amongst the first to be introduced into the market place (de Vos & Simons, 1994). Indeed, in recent years the competent authorities in several EU countries have approved the consumption of such products by professional taste panels as well as targeted consumer groups.

3. Food-grade marker systems

A great variety of approaches have led to the successful development of food-grade marker genes for *L. lactis* and

other lactic acid bacteria. All these marker genes are derived from lactic acid bacteria or other bacteria with a long history of safe use in foods. Food-grade systems that allow cloning and amplification of novel or existing genes have been constructed with these marker genes incorporated into multicopy plasmid vectors derived from lactic acid bacteria (Fig. 1).

Based on the method of selection, the food-grade marker genes can be grouped into dominant or complementation markers. Dominant markers do not rely on specific host genes and hence can be used in all strains belonging to a species and, in many cases, other species of lactic acid bacteria (Fig. 1A). In this respect they can be compared to antibiotic resistance markers. While they are widely applicable and hence desirable, food-grade dominant marker systems are rare, suffer from complicated selection procedures, and, in some cases, comprise several genes and are thus large. In contrast, food-grade complementation markers are dependent on specific mutations in the host chromosome (Fig. 1B). As a consequence, these markers can only be used in specific vector–host combinations. Chromosomal mutations may however be transferred from one strain to another by conjugation, a property which has been exploited to allow the use of a food-grade marker in *L. lactis* (E. Stenby, P. Bruinenberg & W. M. de Vos, unpublished observations). Limited sequence homology may exist between the food-grade marker and the mutated chromosomal gene, but the deleterious effect of this is minimized by constructing appropriate deletions in order to prevent undesired integration by homologous, *recA*-dependent recombination. Complementation markers are usually

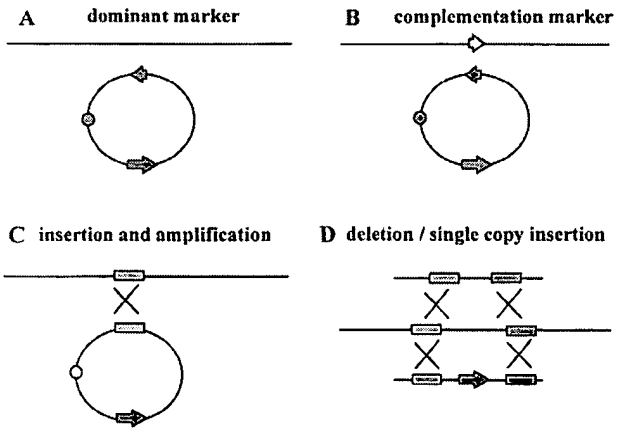

Fig. 1. Schematic representation of the different food-grade markers (A and B) and chromosomal integration strategies (C and D). The chromosome is represented by the single line (A–D). Genes of interest are indicated by the large arrows (A–D). Small arrows indicate food-grade marker genes (A, B); the open arrow is a not functional gene (B). Small circles denote replicons (A–C); open circle indicates a non-functional or conditional replicon (C). Homology areas are indicated with different shading (D).

based on carefully selected genes that encode important and industrially relevant metabolic conversions. As a consequence, their applicability is significant as genes encoding several metabolic functions may be targeted, the size of the complementing DNA may be small, and strong selection pressure may be applied at crucial points in time, contributing to the desired stability (Table 1).

While the distinction between dominant and complementation markers is a useful one, it is practically more convenient to classify the different food-grade marker systems based on the way selection is applied. This allows grouping into three classes discussed below that include markers based on sugar utilization, auxotrophic markers, and markers that confer resistance or immunity (Table 2).

The first class includes both dominant and complementation markers that rely on sugar fermentation. This is a prominent class of markers since lactic acid bacteria have the capacity to utilize a large range of sugars (de Vos, 1996). Three of these are dominant markers that are based on the sugars; xylose, inulin, and sucrose. These sugars are not readily fermented by all species of lactic acid bacteria and hence genes involved in their utilization can be introduced in non-fermenting hosts that do not have any homologues of these genes (Fig. 1A). Xylose fermentation by *Lactobacillus* (*Lb.*) *pentosus* has been thoroughly studied and can be implemented in other lactobacilli, such as *Lb. casei*, by the genes encoding D-xylose isomerase (*xylA*), D-xylulose kinase (*xylB*), and a D-xylose catabolism regulatory protein (*xylR*) (Posno, Heuvelmans, van Giezen, Lokman, Leer & Pouwels, 1991). However, xylose is not fermented rapidly, its addition in pure form may be costly, and the DNA fragment containing the *xylRAB* genes is rather large, limiting the versatility of this food-grade marker. Inulin fermentations depend critically on the activity of levanase. The gene for this extracellular enzyme was isolated from *Bacillus subtilis* and found to be functional in two *Lactobacillus* spp. that secreted an active levanase (Hols, Ferain, Garmyn, Bernard & Delcour, 1994; Wanker, Leer, Pouwels & Schwab, 1995). This food-grade marker may prove to be applicable in various lactic acid bacteria but reportedly suffers from variable production levels and slow fermentation, while depending on a sugar that is not common to many food products. A dominant marker used for *L. lactis* but with potential for other hosts is the one based on the divergent *scrA/scrB* genes of *Pediococcus pentosaceus* that code for sucrose transport via the phosphotransferase system (PTS) and hydrolysis of the resulting sucrose-6-phosphate (Leenhouts, Bolhuis, Venema & Kok, 1998). Sucrose is a cheap and familiar sugar that can be added to many food products. A drawback of the system is the necessity for a high copy number of the *scrA/scrB* genes in order to get sufficient expression for rapid growth of the transformed *L. lactis* strains. However, this property has been used in an

Table 2
Features of food-grade selection marker systems for lactic acid bacteria, including the donors from which the systems have been derived and the hosts that allow their application. The difference between dominant (D) and complementation (C) marker systems is indicated. *Lb*, denotes *Lactobacillus*

Selection marker (D or C)	Donor	Hosts	Selectable marker	Reference
Sugar utilization markers				
Xylose fermentation (D)	*Lb. pentosus*	*Lb. casei*	*xylRAB*	Posno et al. (1991)
Inulin fermentation (D)	*B. subtilis*	*Lb. plantarum* *Lb. casei*	*levA*	Hols et al. (1994); Wanker et al. (1995)
Sucrose fermentation (D)	*P. pentosaceus*	*L. lactis*	*scrA/scrB*	Leenhouts et al. (1998)
Lactose fermentation (C)	*Lb. helveticus*	*Lb. helveticus*	*lacLM*	Hashiba et al. (1992)
Lactose fermentation (C)	*L. lactis*	*L. lactis*	*lacF*	de Vos et al. (1990); MacCormick et al. (1995); Platteeuw et al. (1996)
Auxotrophic markers				
Purine biosynthesis (C)	*L. lactis*	*L. lactis*	tRNA (gln)	Dickely et al. (1995)
Resistance/immunity markers				
Nisin resistance (D)	*L. lactis*	*L. lactis*	Nis^R	Froseth et al. (1988); von Wright et al. (1990); Froseth and McKay (1991); Hughes and McKay (1991); Liu et al. (1996)
Cadmium resistance (D)	*L. lactis*	*L. lactis*	Cd^R	Liu et al. (1996)
Lactacin F immunity (D)	*Lb. acidophilus* *Lb. fermentum* *Lb. gasseri* *Lb. johnsonii*	*Lb. acidophilus*	*lafI*	Allison and Klaenhammer (1996)

advantageous way to achieve amplification in the chromosome (see below).

Two food-grade selection systems are based on the fermentation of lactose, both of these are complementation markers (Fig. 1B). Lactose is a cheap sugar present in various whey-based industrial media. Selection for lactose fermentation is industrially relevant, especially for processes which are dairy related. Pioneering work during the development of transformation systems showed that lactose plasmids could be introduced and selected for in *Streptococcus lactis* (now *Lactococcus lactis*) and *Lb. casei* (Kondo & McKay, 1984; Chassy, 1987). However, these plasmids were large, contained many genes involved in lactose metabolism, and had a low copy number. The lactose complementation systems that were subsequently developed were all based on *lac* genes that were either naturally present or deliberately integrated into the chromosome. A food-grade system based on lactose complementation was described for *Lb. helveticus* where an intact β-galactosidase gene was found to complement a chromosomal mutation in that gene (Hashiba, Takiguchi, Jyoho & Aoyama, 1992). The first system to be described which has been very well-characterized, is based on the small 0.3-kb *lacF* gene, coding for the essential soluble Enzyme III involved in the lactose PTS (de Vos, Vos, Simons & David, 1989, de Vos, Boerrigter, van Rooijen, Reiche & Hengstenberg, 1990). This system builds on the detailed molecular characterization of the lactose fermentation in *L. lactis* (for a review see de Vos & Vaughan, 1994). Lactose-deficient mutants containing a missense mutation in, or complete deletion of, the chromosomal *lacF* gene, were complemented by the wild-type *lacF* gene (de Vos et al., 1990; MacCormick, Griffin & Gasson, 1995; Platteeuw, van Alen-Boerrigter, van Schalkwijk & de Vos, 1996). More importantly, the complementing plasmids were stably maintained during growth on lactose and allowed rapid growth of the complementing strain comparable to that seen in the wild-type. Since their conception and development over a decade ago, the *lacF*-based vectors have been used for the food-grade cloning and expression of a variety of genes. The self-cloning of genes such as *pepN* resulted in 100-fold overproduction of the debittering aminopeptidase N and the design of a controlled lysis system (de Vos & Simons, 1994; Platteeuw et al., 1995; de Ruyter, Kuipers, Meijer & de Vos, 1997).

A second class is formed by the complementation of auxotrophic food-grade markers. An elegant system was developed in *L. lactis* based on suppressor tRNA's (Dickely, Nilsson, Hansen & Johansen, 1995). In this original approach an ochre suppressor tRNA rescued a nonsense mutation in the chromosomal purine genes that are needed during growth of *L. lactis* in milk. Based on this suppressor system, an *L. lactis* strain was constructed by self-cloning carrying multiple copies of the *pepN* gene (Dickely et al., 1995). However, aminopeptidase overproduction was only limited (up to only five-fold), the system can only be applied in specific media,

and the suppressor tRNA may show pleiotropic effects reducing its practical application. A complementation system based on the cloned lactococcal *thyA* gene for thymidilate synthase was also described as a way to select and maintain foreign sequences in *L. lactis* (Ross, O'Gara & Condon, 1990). However, no *L. lactis* strains deficient in the *thyA* gene have so far been reported.

Dominant markers based on resistance or immunity constitute the final class. The first system developed was based on the Nis^R gene coding for a hydrophobic protein that provides resistance to nisin. Various reports on the applicability of this nisin resistance have appeared (Froseth, Herman & McKay, 1988; von Wright, Wessels, Tynkkynen & Saarela, 1990; Froseth & McKay, 1991) including the self-cloning of a phage resistance mechanism in *L. lactis* (Hughes & McKay, 1991). So far the use of this dominant marker in other lactic acid bacteria has not been reported. A specific case is represented by the marker system based on a plasmid encoding cadmium resistance in *L. lactis*. This system alone, or in combination with a Nis^R gene, was proposed as a food-grade marker (Liu et al., 1996). The question however arises as to whether cadmium as a selection marker is desired in a food product. A last dominant marker is based on the *lafI* gene coding for immunity to lactacin F, a bacteriocin produced by *Lb. acidophilus* (Allison & Klaenhammer, 1996). This marker is small and versatile and was found to be applicable in various *Lactobacillus* spp. that show sensitivity to lactacin F. It can be envisaged that a new family of food-grade markers will be developed based on the wealth of data that in recent years has become available on the genetic determinants that confer immunity to antimicrobial peptides produced by lactic acid bacteria (de Vos, Kuipers, van der Meer & Siezen, 1995; Nes, Diep, Havarstein, Brurberg, Eijsink & Holo, 1996).

4. Food-grade chromosome insertion, amplification, and deletion systems

Cloning systems based on plasmids and food-grade markers inherently suffer from instability. This can be prevented or minimized by applying continuous selective pressure such as growing *lacF* complementing plasmids in lactose-containing media. Ultimate stability should be provided by integration into the chromosome. All systems for chromosomal integration are based on non- or conditionally-replicating plasmids that have been well-studied in lactic acid bacteria (de Vos & Simons, 1994). Since several of the non-replicating plasmids are based on replicons of *Escherichia coli* or microbes other than lactic acid bacteria, a specific set of food-grade integration vectors has been developed. These so called Ori^+ vectors only contain the replication origin of the well-characterized *L. lactis* plasmid pWV01 that is only able to replicate when the RepA replication protein is provided in *trans*. Strains of *E. coli* and *L. lactis* were constructed carrying the *repA* gene in their chromosomes that supported replication of the Ori^+ integration vectors (Leenhouts et al., 1996).

Several chromosome delivery systems have been developed to facilitate integration in lactic acid bacteria. Some are based on transposition and may be relatively random (Maguin, Prevost, Ehrlich & Gruss, 1996). Others are based on site-specific recombination (Auvray, Coddeville, Ritzenthaler & Dupont, 1997; Lillehaugh, Nes & Birkeland, 1997). Alternatively, many of the integration systems are dependent on homologous, *recA*-dependent recombination which are discussed below with specific attention to *L. lactis*. The simplest form of chromosomal insertion is obtained when a non-replicating plasmid that has a region of homology with the chromosome is introduced into a host where it integrates via homologous recombination. This way of chromosomal stabilization is known as single cross-over or Campbell integration (Fig. 1C). The efficiency of insertion is dependent on the recombination efficiency of the host as well as the size and sequence of the region of homology that in *L. lactis*, which should be at least 0.4 kb (Biswas, Gruss, Ehrlich & Maguin, 1993). Since recombination events are reversible, single cross-over insertions suffer from an inherent instability that can be overcome by applying continuous selective pressure. This also selects for amplification events caused by insertion of multimers, or due to asymmetric homologous recombination between directly repeated sequences. Food-grade markers have only been used in a few cases to realize these amplifications that may overcome problems relating to a low gene dosage. One such report describes the insertion and amplification of a plasmid carrying the *lacF* marker in the *L. lactis* chromosome during growth on lactose (de Vos, 1988). Another, more recent and complete study describes the use of the *P. pentosaceus* sucrose (*scrA/scrB*) genes to generate amplifications to a level of approximately 20 per chromosome of *L. lactis* (Leenhouts et al., 1998). The stability of these amplified sequences was high, demonstrating the utility this system that combines a food-grade marker and a non-replicating Ori^+ plasmid.

In the case where an integrating plasmid is equipped with two regions of homology that are physically closely linked, two recombination events may result in gene replacement (Fig. 1D). These recombination events may occur simultaneously (double cross-over) or consecutively (single cross-over followed by loop-out deletion). Depending on the nature of the sequences in between the homologous regions, the final result may be a chromosomal deletion or the insertion of a gene of interest. Several gene replacement strategies have been described that allow chromosomal insertions in a food-grade manner which are based on the temperature-sensitive pGhost-series of plasmids, the Ori^+ set of integration vectors and a *lacZ*-based system allowing a blue/white

screening of the second cross-over event (Leenhouts, Kok & Venema, 1991; Leenhouts et al., 1996; Biswas et al., 1993). In some cases, gene replacements are difficult or impossible to obtain. This is observed either when the gene to be replaced is essential, when there are polar effects in the chromosome due to the insertion or expression of the gene of interest, or when a preferred recombination occurs at the homology region where the first cross-over occurred, restoring the wild-type genotype. Deletions as small as four nucleotides have been obtained by gene replacement in the *L. lactis* chromosome (Kuipers, Beerthuyzen, Siezen & de Vos, 1993). Whilst developed for *L. lactis*, most of these approaches can be applied to other lactic acid bacteria and food-grade deletions have been described in *Lb. plantarum* even in an essential gene (Ferain, Garmyn, Bernard, Hols & Delcour, 1994; Hols, Defrenne, Ferain, Derzelle, Delplace & Delcour, 1997). These strains containing 'clean' deletions generated by gene replacement are by definition obtained by self-cloning, considered safe, and can expected to be amongst the first genetically improved lactic acid bacteria to reach the consumer.

5. Food-grade controlled expression systems

Controlled expression systems are important tools that allow genes of interest to be expressed independent of the growth of the production host (Kuipers et al., 1997). Several food-grade inducible expression systems have now been described that allow the induction of gene expression to be modulated in a wide range of lactic acid bacteria (Table 3). While the sugar inducible systems are poorly controlled, several new systems have recently been developed that allow strong control (more than 1000-fold). Two of these expression systems can be induced by food-grade environmental stimuli such as salt and pH, or temperature (Nauta, van den Burg, Karsens, Venema & Kok, 1997; Sanders, Venema & Kok, 1997; Sanders, Leenhouts, Burghoorn, Brands, Venema & Kok, 1998). A special case is the induction of gene expression by bacteriophage infection that results in induction of a phage middle promoter (O'Sullivan, Walker, West & Klaenhammer, 1996). The most versatile system to date is the NICE system (nisin-controlled expression) that has been extensively characterized and responds to externally added nisin and is operating in a variety of lactic acid bacteria (Kleerebezem, Beerthuyzen, Vaughan, de Vos & Kuipers, 1997; Kuipers, de Ruyter, Kleerebezem & de Vos, 1998). The NICE system has been used to design food-grade and self-cloned *L. lactis* strains that lyse upon the addition of nisin and may contribute to the generation of novel flavours in cheese manufacture (de Ruyter et al., 1997).

6. Conclusions

A wide variety of sophisticated systems have been developed for food-grade selection, stable maintenance

Table 3
Food-grade inducible expression systems in lactic acid bacteria, including the donors from which the systems have been derived and the hosts that allow their application. *Lb*, denotes *Lactobacillus*, *Lc*, *Leuconostoc*. The approximate induction factor has been indicated

Induction treatment	Donor	Hosts	Promoter–regulator	Induction factor	References
Lactose fermentation	*L. lactis*	*L. lactis*	*lacA*-LacR	~10	van Rooijen, Gasson and de Vos (1992); Payne, MacCormick, Griffin and Gasson (1996)
Lactose fermentation	*L. lactis*	*L. lactis*	*lacR*-LacR	~10	van Rooijen et al. (1992); Eaton, Shearman and Gasson (1993)
Xylose fermentation	*Lb. pentosus*	*Lb. pentosus*	*xylA*-xylR	~60	Lokman, Leer, van Sorge and Pouwels (1994); Lokman et al. (1997)
Lactose fermentation	*S. thermophilus*	*S. thermophilus*	*lacS*-GalR	~10	Mollet, Knol, Poolman, Marciset and Delley (1993); P. Catzeddu, P. van den Boogert and W. M. de Vos (unpublished results)
Chloride-acid addition	*L. lactis*	*L. lactis*	*gadC*-GadR	>1000	Sanders et al. (1997, 1998)
Temperature upshift	*L. lactis*	*L. lactis*	*tec*-Rro12	>500	Nauta et al. (1996, 1997)
Phage infection	*L. lactis*	*L. lactis*	*φ31*-Tac	>1000	O'Sullivan et al. (1996); Walker and Klaenhammer (1998)
Nisin addition	*L. lactis*	*L. lactis* *Lb. helveticus* *L. casei* *Lc. lactis*	*nisA*-NisR	>1000	de Ruyter, Kuipers, Beerthuyzen, van Alen-Boerrigter and de Vos (1996); Kleerebezem et al. (1997)

and amplification of genes of interest in lactic acid bacteria. In addition, food-grade insertion, deletion and amplification in the chromosome can be accomplished. Finally, controlled gene expression systems have been developed that can be regulated by food-grade stimuli. The availability of these systems begs the question on the timing of their application. The answer relies on political, economic, and emotional considerations rather than on the scientific progress presented here that has resulted in safe and sustainable systems for food-grade fermentations with genetically modified lactic acid bacteria.

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